Universidad del País Vasco
Euskal Herriko Unibertsitatea

ZTF-FCT
Zientzia eta Teknologia Fakultatea
Facultad de Ciencia y Tecnología

Gradu Amaierako Lana / Trabajo Fin de Grado
Biokimika eta Biologia Molekularreko Gradua / Grado en Bioquímica y Biología Molecular

MECHANISMS OF E2F2-MEDIATED TRANSCRIPTIONAL REPRESSION

Egilea/Autor:
Martxel P. Dehesa
Zuzendaria/Director/a:
Dr. Ana Maria Zubiaga

Leioa, 11 de febrero de 2013

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1. Introduction

E2F is a family of transcription factors involved in the cell cycle regulation. They are known to interact with the tumour suppressor protein Retinoblastoma (Rb), and together with it they integrate the Rb/E2F pathway, which is responsible for the regulation of the restriction point R during the G1 phase of the cell cycle (Harbour & Dean, 2000).

According to the current model, during quiesence and the G1 phase of the cell cycle, members of the Rb family of proteins (pRb, p107, p130) are hypophosphorylated and bound to the E2F proteins, and thus inhibiting the transcriptional activity of the E2Fs (Hatakeyama & Weinberg, 1995). The phosphorylation of the Rb family members by the CDK4-6/Cyc D and CDK2/Cyc E throughout the normal progression of the cell cycle causes the release of the E2F transcription factors from Rb. Once the E2Fs are free they can carry out their transcriptional activity, which is necessary for the progression of the cell cycle towards the S phase, activating the transcription of genes required for cell cycle progression, DNA synthesis and replication (**Figure 1**). This makes the Rb/E2F pathway critical to cell cycle regulation and its deregulation can lead to the loss of control in the proliferation and to cancer (Lundberg & Weinberg, 1998).

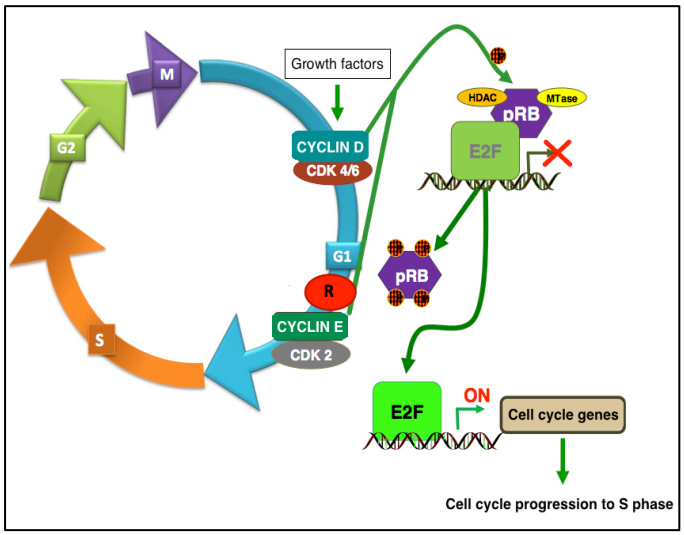

Figure 1: Rb/E2F pathway regulation model.

Despite this generally accepted model, the real picture is a lot more complex. One reason for that is that the E2F family consists of 8 genes from which 10 different protein products have been described, E2F1, 2, 3a, 3b, 4, 5, 6, 7a, 7b and 8.

All of the E2F members have relatively conserved structural domains (**Figure 2**). An example of that is the DNA binding domain (DBD) or the dimerization domain, which is used to bind to DP protein. Additionally E2F7 and E2F8 have two DBD. The Rb binding domain is present in all but the E2F6, E2F7 and E2F8 proteins, and modulates the transcriptional activity of these E2Fs (Chen et al., 2009).

The E2F family of proteins bind to consensus sequences (5'-TTTSSCGC-3' S=C or G) that are present on the promoters of the genes identified as E2F targets, and these targets consists of a set of genes that are essential for cell division, coding for proteins such as Cyc E, Cyc A or RB. They also regulate genes implicated on the biosynthesis of nuclotides (Dhfr, Ts, Tk) and DNA replication (Cdc6, Orc1, and Mcm protein family) (Dyson, 1998).

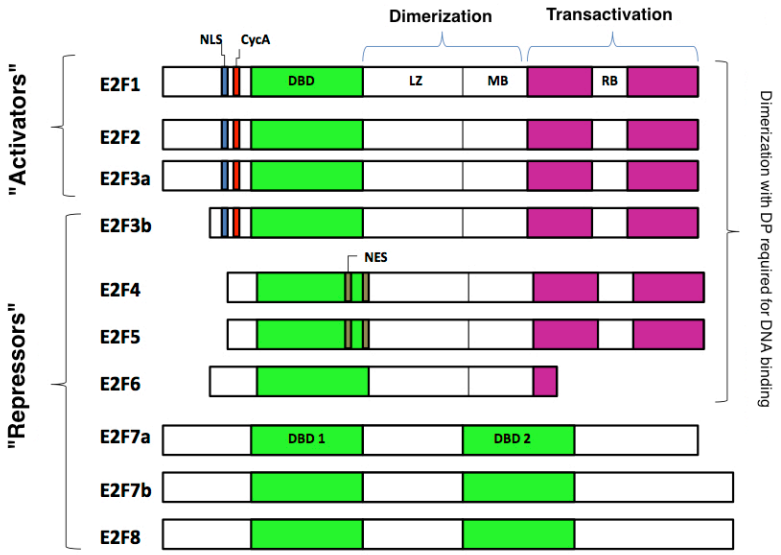

Figure 2: Structure of the E2F family member proteins in mammals. NLS: Nuclear localization signal. CycA: Cyclin A binding domain. DBD: DNA binding domain. LZ: Leucine zipper domain. MB: Marked box. RB: Rb binding domain. NES: Nuclear exportation signal.

Regarding the structure, transcriptional activity and binding partners of the E2F proteins, these have been traditionally divided into "activators" and "repressors" of

transcription. E2F1, 2 and 3a are classified into the activators group whereas the rest of the E2F family (E2F3b, 4,5,6,7,7b,8) are considered repressors. However, this classification is overly simplistic since many studies have shown that one same E2F can act both as an activator and as a repressor in different cellular contexts. The mechanisms for these dual activities have not been defined yet. Moreover, E2Fs regulate each other with positive and negative feedbacks and the targets are redundant among them, increasing the complexity of their regulatory functions (Chen et al., 2009; Murga et al., 2001)

The repression mediated by E2Fs can be of two types based on their interaction with Rb. One of them would be the Rb dependent, where Rb binds to the E2F and induce transcriptional repression inhibiting activation or by binding to the DNA bound E2Fs and recruiting repressor complexes to the target promoters. And the other type of repression would be the Rb independent repression, where the repression is carried out by the E2Fs without Rb acting as a co-repressor (de Bruin et al., 2003). However the interactions between E2Fs and other transcription factors at the target sites is what determines their final role in the transcriptional activity. There are many evidences indicating that some E2Fs, traditionally considered activators, have shown to have also repressor activity, and there are also cases where E2Fs traditionally classified as repressors could be inducing transcriptional activation (Racek et al., 2008).

E2F2 is a good example of a E2F with this dual role in transcriptional activity. E2F2 has been traditionally classified as an activator of transcription, and is commonly found as an activator of various E2F target genes (Muller & Helin, 2000). However, previous results from Dr. Ana Zubiaga's group have shown that it can exhibit both a role as an activator as well as a repressor of target promoters (Infante et al., 2008; Laresgoiti et al., 2013; Osinalde et al., 2013). Examples of these promoters are Chk1, a protein kinase required for checkpoint-mediated cell cycle arrest and Mcm5, a protein from the MCM complex involved in DNA replication. Importantly, both functions are mediated by E2F motifs, and E2F2 was found to act as a Rb independent repressor in HEK-293T cells (**Figure 3**).

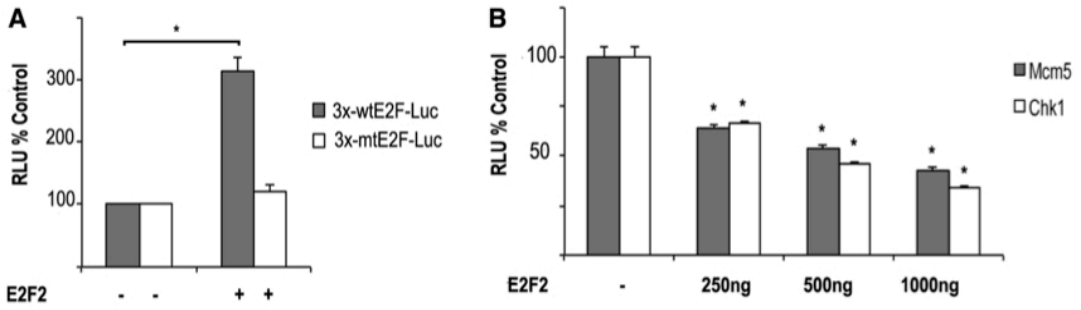

Figure 3: Luciferase results showing E2F2-induced activation of a promoters with 3xE2F sites and repression of transcription driven by Mcm5 and Chk1 promoters in HEK-293T cells, which also contain E2F sites (Laresgoiti et al., 2013).

Considering that E2F2 has been observed to act traditionally as a transcriptional activator, it is reasonable to think that perhaps E2F2 could be activating the expression of a member of the E2F family, and thus, be mediating the E2F2 repressor activity indirectly.

1.1 Aim of the study

In this work we wanted to study the mechanism of E2F2-mediated repression. Our hypothesis is that E2F2 activates the expression of one or more E2F members of the "repressor" subset of the family through the E2F motifs present in their promoters, and those repressor E2F(s) would subsequently repress the target promoters.

To address this hypothesis, we focused on E2F7. E2F7 is a repressor that lacks the Rb binding domain, and associates with DNA through E2F binding sites (de Bruin et al., 2003). Furthermore, E2F7 itself is also regulated by E2F motifs on its own promoter, and it has been shown to repress DNA metabolism and replication genes in late S-phase (de Bruin et al., 2003; Westendorp et al., 2012). E2F7, together with E2F8 has been found to form heterodimers, being critical on cell proliferation and development, and both seem to have similar functions (Li et al., 2008).

Preliminary results from Zubiaga's group have indicated that E2F2 activates E2F7 transcription in U2OS cells, suggesting that E2F2's repressor function could be

mediated by E2F7. For this purpose, we focused on studying E2F7's role on the target genes previously known to be repressed by E2F2: Chk1 and Mcm5.

The specific aims for this work were the following:

- Confirm that E2F2 induces E2F7 in HEK-293T cells
- Assess whether E2F7 acts as a transcriptional repressor on E2F sites
- Evaluate the role of E2F7 on E2F2-mediated transcriptional repression of Chk1 and Mcm5.

2. Materials and methods

2.1 Cell culture conditions

Human embryonic kidney (HEK) 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% of fetal bovine serum (FBS) at 37ºC and 5% CO_2 in 100mm plastic plates. The cell line contains the SV40 large T antigen, which is known to be a strong inhibitor of Rb (DeCaprio et al., 1988).

2.2 Transfections and siRNA-mediated knockdown

Transient transfections were performed by the calcium phosphate method (Kingston, Chen, & Okayama, 2001). Cells were cultured in 6-well plates until the cell density was 40-50% of the plastic surface. One hour before the transfection 2ml of new media was added to the cells (DMEM+10% FBS). The DNA plasmid mixture was prepared in a 200µl $CaCL_2$ 250mM solution with 1-2µg DNA for each well. Then an equal volume of HBS 2X (50mM HEPES, 1.5mM Na_2HPO_4, 140mM NaCl adjusted to 7.05 pH) was added and mixed. A total volume of 400µl of the mixture were added to each well and cells were incubated for 12h at 37ºC and 5% CO_2. Then cells were washed with Phosphate Buffered Saline (PBS) and new media was added (DMEM+10% FBS) incubating the cells for a total of 48h since transfection before further analysis was done. For knockdown of endogenous genes, siRNA oligonucleotides were transfected at a final concentration of 10nM using 2µ

Lipofectamine RNAiMAX (Life Technologies) in a transfection volume of 300µl/well opti-MEM (Life Technologies) and incubated for 20 minutes before addition to the cells. Silencing of E2F7 gene was performed with siE2F7 (s44665, Ambion) and as a control a non-targeting negative control siRNA was used (Ambion *Silencer* Negative Control #1). Plasmid vector containing E2F2 gene inserted was pRc/CMV 5.4Kb (Invitrogen), pRc/CMV-HA-E2F2 was same as previously used in Zubiaga's group (Laresgoiti et al., 2013). For E2F7 over-expression pCMV6-XL5 4.7Kb plasmid vector was used.

2.3 Luciferase activity assays

For luciferase activity assays, cells were transfected with 200ng of the firefly luciferase reporter vector and 20ng of the *Renilla* luciferase reporter vector (pRT-LK) with the described calcium phosphate transfection method. The reporter firefly luciferase activity was normalized to the transfection efficiency estimated by the activity of *Renilla* luciferase in each sample (Dual-Luciferase Reporter Assay System, Promega). The reporter plasmid constructs p3xE2F-Luc, was the luciferase gene under a synthetic promoter containing three canonical E2F motifs (tttcgcgcca). pChk1-Luc (-613 to +1664 genomic region of Chk1 gen promoter) containing 6 predicted E2F sites. And the Mcm5-Luc (-528 to +131) of the murine *Mcm5* genomic region, containing 2 predicted E2F sites, were used as previously in Zubiaga's group (Laresgoiti et al., 2013).

2.4 Gene expression analysis

Gene expression levels were measured at mRNA levels for E2F7 by quantitative polymerase chain reaction (RT-qPCR) and at protein level by Western blot. Total RNA was extracted from the cells with TRIzol reagent (Invitrogen), and was purified using RNeasy Mini Kit (Quiagen). Purified RNA was resuspended in RNase free water and the RNA samples were then quantified at A260 with Nanodrop (Thermo Scientific) spectrophotometer. 300ng of each sample were then used for reverse transcription PCR (RT-PCR) in order to obtain cDNA with the High-Capacity cDNA

RT Kit (Applied Biosystems) following the manufacturer's recommendation. qPCR was performed on 1:30 cDNA dilutions and 1x Power SYBR Green PCR Master Mix (AB). Primers used for amplification of E2F7 and housekeeping gene hHPRT were same as described previously (Laresgoiti et al., 2013). Reaction was 40 cycles (15´´ 95ºC and 1´60ºC) with initial 10´at 95ºC. Standard curves were calculated using cDNA dilutions to determine the linear range and efficiency of each primer pair. Reactions were done in triplicate, and relative amounts were normalized to the internal control hHPRT.

In Western blot analyses protein was extracted from the cells using a buffer with 500mM $NaPO_4H$, pH 7.2; 500mM EDTA, 500mM EGTA, 500mM NaCl, 500mM NP-40, 100mM ß-Glucophosphate, 100mM PMSF, 200mM Na_3VO_4 and a cocktail of protease and phosphatase inhibitors (Roche). Protein concentration from each sample was determined using DC Protein Assay (Bio-Rad) with a BSA standard curve. 20µg of protein were loaded on each lane of the Sodium dodecyl sulphate-polyacrylamide gels (SDS-PAGE) in 8%-12% Acrylamide gels that were run at 100V and transferred onto a nitrocellulose membrane also at 100V (Bio-Rad). Proper transference of the protein to the membrane was confirmed staining the membrane with ponceau. The membranes were then washed with TTBS (Tris 50mM, NaCl 150mM, 0.5% tween 20) and blocked with 5% skimmed milk in TTBS for one hour. Primary antibodies rabbit anti-E2F2 (diluted 1:400 sc-633) goat anti-E2F7 (diluted 1:400 sc-32574) and for endogenouscontrol mo use anti-α-tubulin (diluted 1:5000 sc-*T9026*) from Sigma were incubated over night in 10ml of 5% skimmed milk in TTBS. Immunocomplexes were visualized with horseradish peroxidase-conjugated anti-mouse anti-rabbit or anti-goat IgG antibodies (Santa Cruz) by chemiluminiscence detection with ECL (Santa Cruz) with a ChemiDoc camera (Bio-Rad).

3. Results

3.1 E2F2 activates E2F7 transcription

First of all, I wanted to see whether E2F2 activates transcription of E2F7 in our cellular model, the HEK-293T cells, a point that has not been previously tested.

In the HEK-293T cells, the endogenous expression of E2F2 is low. This allows us to assess the transcriptional effect of the ectopic expression of this factor. In order to test this, I transfected the cells with a plasmid containing the E2F2 gene under the control of a constitutive citomegalovirus promoter (CMV). Subsequently, the RNA and the proteins were extracted from the cells. RT-qPCR and Western blot assays showed low levels of basal E2F7 expression, but over-expression of E2F2 resulted in an increase of E2F7 levels, both at the mRNA as well as protein levels. Quantification of the data showed E2F7 expression to be up-regulated around 60% at the mRNA level, and 30% at the protein level. As a control, we also examined E2F2 expression in parallel (**Figure 4**).

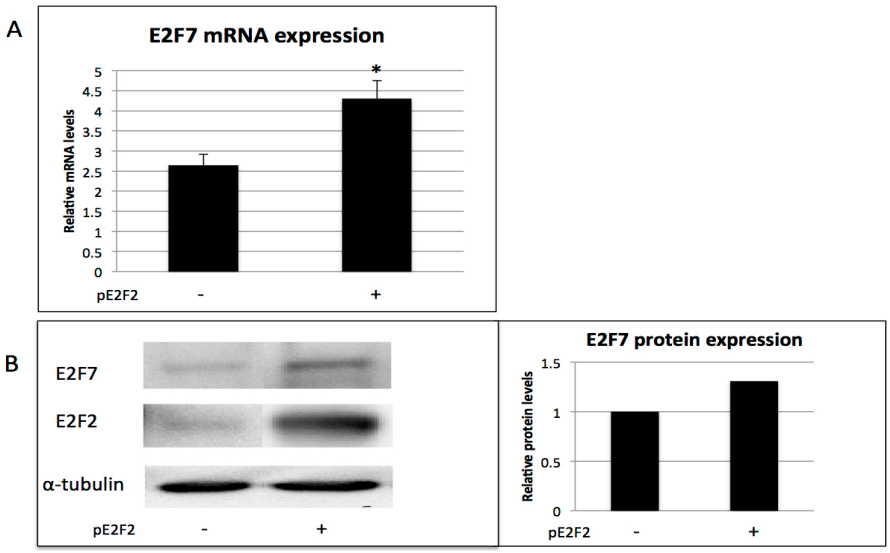

Figure 4: Over-expression of E2F2 induces E2F7 expression both at mRNA and protein levels. (A) mRNA expression levels of E2F7. HEK-293T cells were transfected with pE2F2 or the empty vector pRc/CMV. RT-qPCR analyses were carried out with the mRNA extracted 48h after transfection. The mRNA levels were compared to endogenous HPRT mRNA levels. The values shown represent the mean ±SE (n=3 independent experiments) (*$P<0.05$) (B) Protein levels of a Western blot analysis for E2F7, E2F2 and endogenous control alpha-tubulin. HEK-293T cells were transfected as explained above. Band quantification was performed with image analysis software measuring pixel density. E2F7 bands were normalized with alpha-tubulin and are shown as fold change over the bands transfected with the empty vector.

3.2 E2F7-mediated transcriptional repression

After demonstrating that E2F2 activates E2F7 transcription, we proceeded to assess the transcriptional role of E2F7 on target consensus E2F sites.

In order to do this, we used an approach where the transcriptional activity is detected by the expression of a reporter gene. In our case, the luciferase activity assay. The cells were transfected with a vector containing the E2F7 factor in order to over-express it, or instead, with a siRNA oligonucleotide to silence endogenous expression of E2F7. In both cases, a vector containing the firefly luciferase gene under a promoter harbouring three canonical E2F sites (tttcgcgcca) was co-transfected to assess E2F7 activity (**Figure 5**).

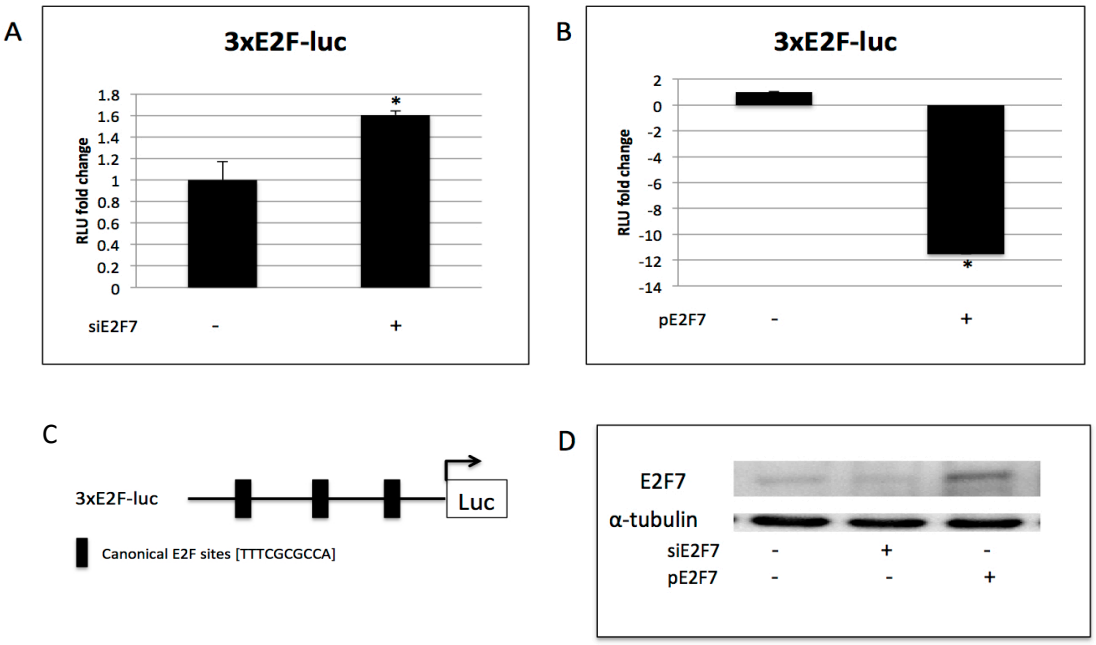

Figure 5: E2F7 acts as a transcriptional repressor of the luciferase reporter containing a synthetic promoter with three canonical E2F sites. (**A**) When E2F7 is silenced the transcriptional activity of the reporter is significantly increased. (**B**) When E2F7 is over-expressed transcriptional activity of the same synthetic promoter is significantly decreased. (**C**) Schematic representation of the 3xE2F-luc reporter construct with three consensus E2F sites. (**D**) The Western blot bands confirm the correct over-expression and silencing of E2F7, using indicated antibodies. HEK-239T cells were transfected with 200ng of the indicated luciferase reporter construct and 1000ng of pE2F7 over-expression vectors or 10nM of the siRNA oligonucleotides. The cells were co-transfected with 20ng of a plasmid containing *Renilla* luciferase to normalize luciferase activity accounting for transfection efficiency. Luciferase activity (RLU) is presented as a ratio of *firefly/Renilla* intensities. Data are shown as fold change over the empty pRc/CMV transfection or the nonspecific siRNA respectively. The values shown represent the mean ±SD (n=3 independent experiments) (*$P<0.05$).

With this setup, when the cells were transfected with the siE2F7 oligonucleotides, we saw an increase of around 60% in the luciferase activity for the synthetic E2F target promoter compared to the control. This indicates that removal of E2F7 allows higher expression of the promoter (**figure 5A**). When the cells were transfected with the same luciferase vector but with pE2F7 over-expression vector instead, we could detect a decrease of eleven fold change in the reporter activity (**figure 5B**). Both types of approaches confirm that E2F7 plays a role as a repressor for this kind of promoter with E2F consensus sites.

3.3 E2F7 is responsible for part of E2F2-mediated repression

E2F2 has a well-known role as transcriptional activator. However, as mentioned before, there are cases where it has shown a role as a transcriptional repressor. Taking into account the results we got from the experiments where we saw that E2F2 induces transcription of E2F7 and that E2F7 functions as a transcriptional repressor, we wanted to assess the role of E2F7 on E2F2-mediated repression. Specifically, we wanted to see if E2F7 could be driving E2F2-mediated repression of Chk1 and Mcm5 genes. The expression of these genes had been previously shown to be repressed by E2F2 in a recent publication (Laresgoiti et al., 2013). In these experiments, we used luciferase reporter constructs under the control of promoters of these two genes to assess transcriptional activity.

The Mcm5 promoter region is a promoter that contains two predicted E2F consensus sites, one of them is located at -212 and the other site is at -7. Chk1 promoter of the reporter construct has six E2F predicted consensus sites located at +21, +129, +609, +713, +814, and +1163 (**Figure 6A)**.

We first examined whether the basal activity of these genes was regulated by E2F7. When siE2F7 oligonucleotides were transfected into the cells to silence E2F7 expression, luciferase activity for both Mcm5 and in a stronger manner for Chk1 reporter was increased, compared to the control transfected with the nonspecific siRNA. This suggests us that E2F7 has also a repressor role on these promoters.

Moreover, it seems that in normal conditions, E2F7 is already repressing the promoters and that if we silence it, their expression is increased (**Figure 6B**).

In order to test whether E2F7 was being responsible for E2F2-mediated repression, we transfected the cells to up-regulate E2F2 and repress the promoters of the target genes, as well as to silence E2F7 expression to assess the effect on the expression of the genes.

Transfecting the cells with the over-expression vector pE2F2 along with the luciferase reporter for Mcm5 or Chk1, led to a 40% reduction in luciferase activity compared to the controls, consistent with the described activity of E2F2 regulating these genes. However, when together with E2F2 over-expression, we transfected siE2F7 to silence its expression, the luciferase activity seen for Chk1 was back to levels similar to the control, and in the case of Mcm5, the inhibition of the repression could be detected in a subtle but significant way. This indicates that without E2F7, E2F2 cannot induce the transcriptional repression on these promoters completely, pointing at E2F7 as a mediator in the repression induced by E2F2 (**Figure 6C**).

However, if E2F7 was the only mediator of the E2F2 induced repression, we would expect that when E2F7 is silenced and E2F2 over-expressed, the luciferase activity would be at least as high as when E2F7 is silenced, which would be higher than the control. What we see when we over-express E2F2 and silence E2F7, is that the inhibition of the repression mediated by E2F2 is only partial. This suggests to us that E2F7 is carrying out the E2F2-mediated repression, but only partially.

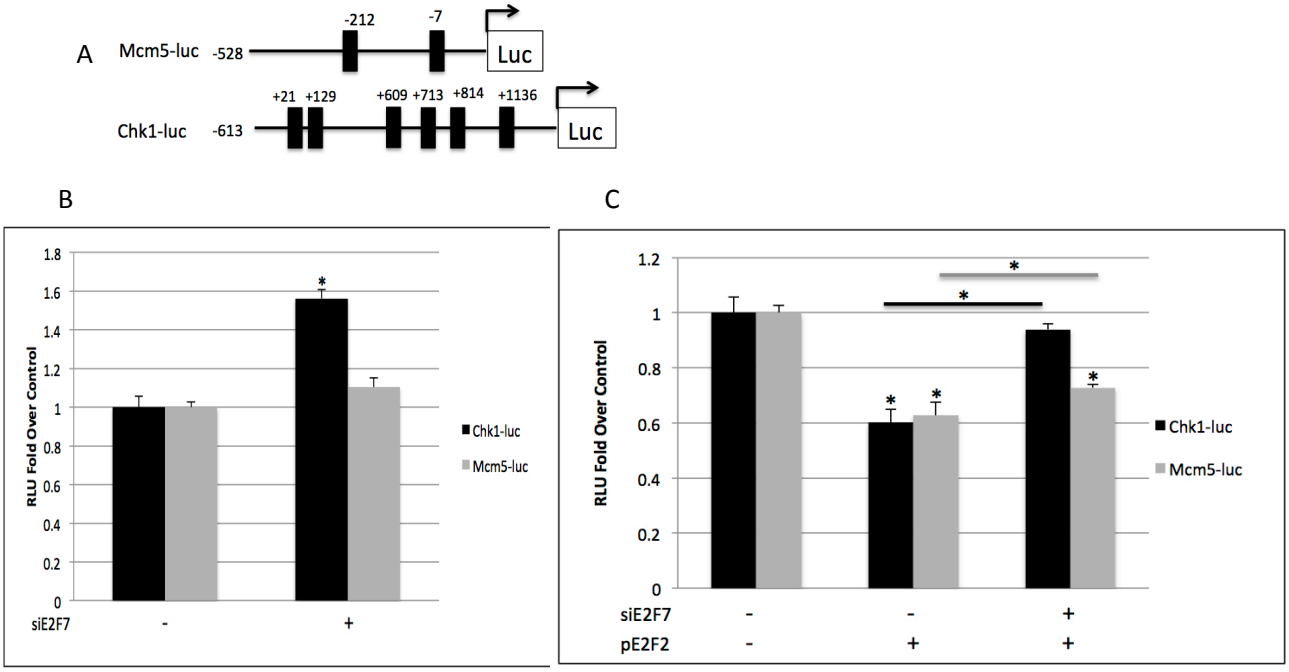

Figure 6: E2F7 represses E2F target genes Chk1 and Mcm5 and is responsible for part of the transcriptional repression induced by E2F2. (A) Schematic representation of mouse *Mcm5* and human Chk1 regulatory regions cloned upstream of the luciferase transcriptional unit. Boxes indicate predicted E2F consensus binding sites. (B) E2F7 is repressing transcription driven by Chk1 and Mcm5 promoters, which is enhanced when E2F7 is silenced using siRNA oligonucleotides. (C) Overexpression of E2F2 leads to the repression of those same promoters, down to 40% compared to the control transfected with empty vector and nonspecific siRNA. When co-transfection of pE2F2 and siE2F7 is given, the repression induced by E2F2 overexpression is recovered to control levels in Chk1-luc and partially recovered in a significant manner for Mcm5-luc. HEK-239T cells were transfected with 200ng of the indicated luciferase reporter construct and 500ng of pE2F2 in the cells co-transfected with Chk1-luc or 250ng of pE2F2 in the cells co-transfected with Mcm5-luc. The cells were co-transfected with 20ng of a plasmid containing *Renilla* luciferase to normalize luciferase activity accounting for transfection efficiency. Luciferase activity (RLU) is presented as a ratio of *Firefly/Renilla* intensities. Data are shown as fold change over the empty pRc/CMV and nonspecific siRNA control transfection. The values shown represent the mean ±SD (n=3 independent experiments) (*$P<0.05$).

4. Discussion and further work

From the results presented above, we can gain insight into the type of mechanisms that are used in the transcriptional regulation in the E2F family. It seems clear from our results that E2F2 is activating the transcription of E2F7, probably trough E2F sites that are present on its promoter.

Putting it into a cellular context, these results fit with the proposed progression of events during the cell cycle, where the classical E2F activators, such as E2F2, carry out their transcriptional activity at the G1 and S phases of the cell cycle, activating the genes necessary cell cycle progression. E2F activators (E2F1-3) would also induce the expression of E2F repressors such as E2F7, 8 and 6. In S phase, their role would be to target the same response genes in a latter phase in order to silence their expression, and thereby, allow cell cycle progression (**Figure 7**).

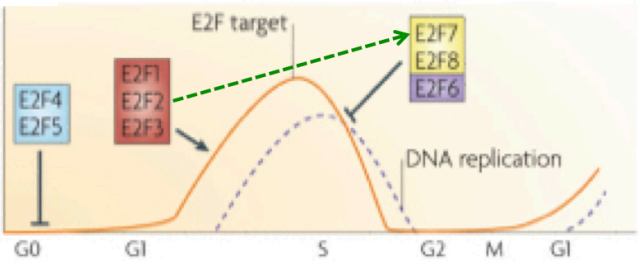

Figure 7: The activity of E2F members during the cell cycle. E2F activators would induce E2F repressors to act in the S phase over common targets (Chen, Tsai, & Leone, 2009).

Regarding the transcriptional activity of E2F2 and E2F7, our results indicate that they both can function together on the same canonical E2F sites. Based on our results from the use of the synthetic promoter with the three E2F sites as well as the natural Chk1 and Mcm5 promoters, E2F7 would be a clear transcriptional repressor. Regarding E2F2-mediated repression, we have demonstrated that E2F7 is responsible for E2F2-mediated repression, at least partially. Therefore, this would be one indirect mechanism by which E2F2 induces repression. Whether E2F2 can also participate directly in the transcriptional repression remains to be elucidated.

An interesting experiment for the future would be to test in similar experiments E2F7 together with E2F8. These two E2F repressors are similar in function and structure, and they have been found to act together as heterodimers, playing critical roles in cell cycle and development. Considering E2F8's similarity to E2F7, there is a chance that this protein also takes part in the mentioned indirect mechanism, which would expect to enhance the E2F2-mediated repression. Role of E2F6 in this mechanism should also be evaluated, due to its known role as repressor. Complementary methods such as chromatin immuno-precipitations would also be useful to better

understand the location and level of occupancy of the E2Fs and other transcription factors on the promoters. Aside from the transcription factor regulation, there is also another level of regulation for the target genes at the RNA level, where miRNAs of other genes could also regulate E2F target genes, and give to the regulation of the cell cycle genes another level of complexity.

A better understanding of the E2F regulation network and their biological implications would allow to increase our understanding of complex processes such as the cell cycle and pathologies like cancer.

4.1 Conclusions

- E2F2 induces expression of E2F7 in HEK-293T cells
- E2F7 is a transcriptional repressor of E2F target promoters
- E2F7 is partially responsible for E2F2-mediated transcriptional repression of Chk1 and Mcm5 promoters.

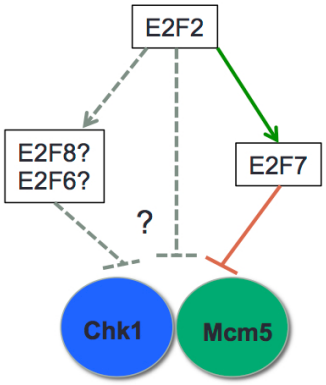

Figure 8; Model for the possible transcriptional regulation in the E2F2-mediated repression. Continuous lines have been demonstrated in this work and dashed lines represent possible complementary mechanisms.

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